

RHAMNOLIPIDS PRODUCED BY *PSEUDOMONAS AERUGINOSA*
GROWN ON *n*-PARAFFIN
(MIXTURE OF C₁₂, C₁₃ AND C₁₄ FRACTIONS)

SEIGA ITOH, HARUO HONDA, FUSAO TOMITA and TAKEO SUZUKI

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.,
Machida, Tokyo, Japan

(Received for publication August 27, 1971)

Two kinds of glycolipids (R-1 and R-2) were produced in the culture media by several strains of *Pseudomonas aeruginosa* when grown on *n*-paraffin (mixture of C₁₂, C₁₃ and C₁₄ fractions). These compounds were isolated through the extraction of the culture broth with ethylacetate and the chromatography on silicic acid column. On the basis of chemical analysis, these lipids were characterized as 2-O- α -L-rhamnopyranosyl- α -L-rhamnopyranosyl- β -hydroxydecanoyl- β -hydroxydecanoate (R-1) and L- α -rhamnopyranosyl- β -hydroxydecanoyl- β -hydroxydecanoate (R-2). Bactericidal activity of R-2 which is postulated to be a precursor of R-1 was remarked against gram-positive bacteria. Further, these compounds also demonstrated mycoplasma-cidal and antiviral activities *in vitro*.

It has been known that a rhamnolipid was produced by *Pseudomonas aeruginosa*, which was characterized as 2-O- α -L-rhamnopyranosyl- α -L-rhamnopyranosyl- β -hydroxydecanoyl- β -hydroxydecanoate^{1,2,3}. Although the study on the biosynthesis of this lipid suggested the presence of another rhamnolipid as a precursor of the former, no report is concerned with the extracellular production and isolation of this lipid. In addition there is little report about the biological activities of these lipids.

In the course of screening studies, we found that *Pseudomonas aeruginosa* grown on *n*-paraffin as the sole source of carbon produced considerable amounts of mycoplasma-cidal substances. These were isolated and characterized as rhamnolipids (R-1 and R-2). R-1 was the known rhamnolipid, while R-2 was the newly isolated compound which was postulated as the precursor of the former.

The present paper deals with the isolation of these rhamnolipids and their biological activities. Culture conditions essential for rhamnolipids production were also described.

Materials and Methods

1. Microorganisms and Culture Conditions

Pseudomonas aeruginosa KY 4025, KY 3977, KY3997 and I-85, were used through this work. Each microorganism was cultivated in the following medium: *n*-paraffin 10 % v/v, KH₂PO₄ 0.1 %, Na₂HPO₄·12H₂O 0.1 %, MgSO₄·7H₂O 0.03 %, ammonium sulfate 0.4 %, corn steep liquor 0.2 %, yeast extract 1.0 %, FeSO₄·7H₂O 0.01 % in tap water. The pH was adjusted to 6.3.

For the production of glycolipids, 30-liter jar fermentor containing 15 liters of the

medium was used under the condition of agitation of 450 r.p.m. and aeration of 15 liters per minute at 30°C. The inoculum was prepared by the previous method⁴) and transferred to the growth medium at the ratio of 5~10% by volume. The pH of the medium was adjusted to pH 6.0~6.5 with ammonia water during incubation.

2. Estimation of Biological Activities

Antibacterial activity was estimated by a conventional paper disc method using *Bacillus subtilis* ATCC 6633 grown on a nutrient agar at pH 7.0 as a test organism.

Mycoplasmacidal activity was also assayed by the similar method using *Mycoplasma laidlawii* and *M. gallisepticum* as test organisms. *M. laidlawii* was kindly supplied by Dr. N. ISHIDA, School of Medicine, Tohoku University. The culture medium for *M. laidlawii* was prepared by mixing the following nutrients autoclaved separately; Trypticase Soy Broth (BBL, Dixon & Co.) 3 g dissolved in distilled water 90 ml, yeast extract 10 ml prepared from Nitten Dry Yeast (Nippon Beet Sugar Manufacturing Co.) 2.5 g, thallium acetate 50 mg and glucose 1.0 g. Penicillin G was also added to the medium at the ratio of 50 units/ml. *M. gallisepticum* strain KP-13 and 333P (spiramycin-resistant) were kindly supplied by Dr. C. KUNIYASU (National Institute of Animal Health). The culture medium for *M. gallisepticum* was prepared by mixing the following components sterilized separately; PPLO Medium (Eiken Chemical Co.) 17.3 g dissolved in distilled water 1,000 ml, horse serum 200 ml, penicillin G (10,000 units/ml) 10 ml. The stock culture was maintained at -80°C in the freezer.

Antiviral activity was estimated by the degree of suppression of cytopathic effect of viral infected monolayers. Confluent cell monolayer sheets were obtained in test tubes by incubating typsinized 10~12 days chick embryo in HANKS' saline (Nissui Seiyaku Co.) supplemented with calf serum 2% and lactalbumin hydrolysate 0.5% (Difco Laboratories Inc.) at 37°C for 3 days. After removal of the medium, the monolayers were infected with 50 TCID₅₀ of vaccinia virus in HANKS' saline containing calf serum 1%, lactalbumin hydrolysate 0.5% and given amounts of samples. After the incubation of the infected cultures at 37°C for 2 days, cytopathic effect was examined by direct microscopic observation. Virus was kindly supplied by Dr. N. ISHIDA of Tohoku University.

3. Determination of Glycolipids

Two sorts of glycolipids produced by this microorganism were assayed by the following procedure: Samples (1 ml) of the culture broth were extracted with ethylacetate (5 ml). A given volume of the extract was evaporated on boiling water bath and subjected to the colorimetric determination of sugars by anthrone sulfate or phenol sulfate method^{5,6}). The linear correlation was demonstrated between the quantity of rhamnolipid (R-1 or R-2) and optical density determined by the above methods. Total amounts of glycolipids were calculated as R-1 using the crystalline R-1 as the standard.

4. Paper and Thin-layer Chromatography

Toyoroshi No. 51A papers and silica gel G plates were employed for this work. The following solvent systems were used for paper chromatography; butanol-pyridine-water (6:4:3) and ethyl acetate-isopropanol-water (16:6:3), and for thin-layer chromatography; chloroform-methanol-acetic acid (80:15:5) and *n*-hexane-ether-acetic acid (80:15:5). Rhamnolipids were detected on plates by heating after spraying anthrone reagent. Free sugars were detected by anisidine reagent or alkaline silver nitrate reagent.

Results and Discussion

1. Isolation of Glycolipids, R-1 and R-2

When *Pseudomonas aeruginosa* KY 4025 was incubated with *n*-paraffin as the sole source of carbon, glycolipids appeared in the culture medium slightly later than the cell growth occurred and increased with the time. From 15 liters of the culture broth obtained after 55-hour incubation in 30-liter jar fermentor, the isolation of

these lipids were attempted. A typical isolation procedure is given below for a starting volume of 5 liters of the broth.

The fermentation broth adjusted to pH 5.0 was extracted with ethylacetate (10 liters) twice at room temperature and the combined extract was evaporated at 40°C *in vacuo* after dehydration with sodium sulfate. The residue dissolved in 0.05 M sodium bicarbonate solution. The aqueous solution was adjusted to pH 4.0 and extracted again with ethylether. After the extract was concentrated to small volume (200 ml), it was submitted to the chromatography on a 24 cm² × 33 cm column of silicic acid (Mallinckrodt Chemical Works Limited).

n-Paraffin was first separated by eluting with excessive amount of *n*-hexane, and then chloroform was used to flow out yellow pigment which was estimated as phenazine-1-carboxylic acid. Subsequently, one of the glycolipids (R-2) was eluted with chloroform-methanol (97:3) and another lipid (R-1) was finally flowed out with chloroform-methanol (93:7). Elution profile of the silicic acid chromatography is shown in Fig. 1.

R-1 fraction having reddish brown coloration was concentrated to dryness. After the residue was taken out with alkaline solution, it was acidified to pH 3.0~4.0 by dropwise addition of HCl. White scales came out after keeping the resulting turbid solution for a few hours at room temperature. These were recrystallized by

Fig. 1. Silicic acid column chromatogram of rhamnolipids produced by *Pseudomonas aeruginosa*.

Column: 24 cm² × 33 cm

Eluant: The mixture of chloroform and methanol, and ratio of methanol was altered at the range of 0~10 %.

- Rhamnolipids determined by phenol-sulfate method.
- Antibacterial activity determined by a conventional paper disc method using *B. subtilis* ATCC 6633 as a test organism.

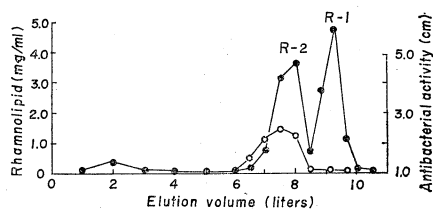


Table 1. Effect of carbon source on rhamnolipids production by *Pseudomonas aeruginosa*

Strain	Carbon source		Rhamnolipids production* (mg/ml)
KY 4025	<i>n</i> -Paraffin**	10 %	8.5
KY 4025	Glycerol	10 %	5.5
KY 4025	Glucose	4.5 %	0.7
I-85	<i>n</i> -Paraffin**	10 %	3.2
I-85	Glycerol	10 %	4.7
I-85	Glucose	4.5 %	1.3

The incubation was performed using 2-liter Erlenmeyer flasks containing 300 ml medium on a rotary shaker at 30°C for 6 days.

* Total amounts of rhamnolipids produced in the culture broth was determined as described in Materials and Methods and calculated as R-1.

** In the case of hydrocarbon fermentation the baffle plates were equipped inside of flasks.

n-Paraffin was mainly composed of C₁₂, C₁₃ and C₁₄ fractions.

Table 2. Antimicrobial spectra of rhamnolipids, R-1 and R-2

Test organisms	M.I.C. (mcg/ml)*	
	R-1	R-2
<i>Escherichia coli</i> (multiple drug resistant)	>1,700	>833
<i>Streptococcus faecalis</i> ATCC 10541	>1,700	2.6
<i>Pseudomonas aeruginosa</i> BHM 1	>1,700	>833
<i>Staphylococcus aureus</i> ATCC 6538	1,700	5.2
<i>Escherichia coli</i> ATCC 26	>1,700	>833
<i>Bacillus subtilis</i> 10707	208	5.2
<i>Proteus vulgaris</i> ATCC 6897	>1,700	(5.2)**
<i>Shigella sonnei</i> ATCC 9290	>1,700	>833
<i>Salmonella typhosa</i> ATCC 9992	>1,700	>833
<i>Klebsiella pneumoniae</i> ATCC 10031	>1,700	>833

* Minimal inhibitory concentrations of rhamnolipids by agar dilution method.

** Calculated from minimal concentration of partial inhibition of growth.

repeating the same procedure as above. Purified crystals (R-1, 3.7 g) were obtained.

R-2 fraction having brown coloration was similarly concentrated. The residue was dissolved in ethylacetate and washed with a small volume of acidic water. The solvent layer dehydrated was subjected again to chromatography of silicic acid column according to the same way as above. As the results, the colorless materials (R-2, 3.5 g) was obtained. The powder of R-2 was also obtained if the solution of R-2 in 0.05 M sodium bicarbonate was lyophilised.

Both the glycolipids were soluble in methanol, chloroform, ethylacetate, ethylether and sodium bicarbonate solution but not soluble in water or *n*-hexane.

2. Characterization of Glycolipids

R_f values of these lipids on thin-layer chromatogram were 0.40 for R-1 and 0.80 for R-2, when chloroform-methanol-acetic acid (80:15:5) was used as a developing solvent. The color reaction of both isolated lipids with anthrone reagent suggests the presence of sugar moiety as the constituent of the lipids. In an attempt to characterize the sugar moiety of R-1 firstly, it was subjected to hydrolysis with 1 N sulfuric acid on boiling water bath for 2 hours and the resultant was treated with ethylether. An aqueous phase of hydrolysate gave a free monosaccharide, which indicated the identical behavior with authentic L-rhamnose on paper chromatography. On the other hand, ether extract of the hydrolysate displayed a single spot on thin-layer chromatogram using the solvent, *n*-hexane-ether-acetic acid (80:15:5). The lipophilic material was isolated from this extract by silicic acid column chromatography and characterized as hydroxyfatty acid by NMR and mass spectra. The details of these physico-chemical analyses will be published by our co-workers⁷⁾. In the other experiment, mild hydrolysis of R-1 with 0.5 N sodium hydroxide gave another glycolipid and more lipophilic material; the former gave also L-rhamnose and hydroxyfatty acid by hydrolysis with 1 N sulfuric acid and the latter was identical fatty acid with that indicated above.

To survey chemical constituents of R-2, similar hydrolysis with acid or alkali was carried out and the hydrolysate was examined by paper and thin-layer chromato-

Table 3. Mycoplasmacidal activity of R-1, R-2 and some antibiotics

	<i>M. laidlawii</i> (cm)	<i>M. gallisepticum</i>	
		KP-13 (cm)	333 P (cm)
R-1 (250 mcg/ml)	2.6	0	0
R-1 (1 mg/ml)	5~6	0	0
R-1 (10 mg/ml)	N. T.	2.0	1.7
R-2 (1 mg/ml)	‡	0	0
R-2 (10 mg/ml)	N. T.	2.3	2.2
Chloramphenicol (100 mcg/ml)	0	1.8	1.8
Spiramycin (10 mcg/ml)	1.4	2.3	0
(100 mcg/ml)	2.2	3.2	1.1

Mycoplasmacidal activity was assayed by a conventional paper disc method. The numerals show the diameter of inhibitory zone against the test organism. N. T. : Not tested.

Table 4. Antiviral activity of R-1 and R-2.

	Concentration (mcg/ml)					
	100	50	25	12.5	6.25	3.125
R-1	Toxic	±	±	±	+	‡
	Toxic	±	±	±	‡	‡
	Concentration (mcg/ml)					
	100	20	4	0.8	0.16	
R-2	Toxic	±	+	‡	‡	
	Toxic	±	‡	‡	‡	

Antiviral activity was estimated using vaccinia virus *in vitro* (see Materials and Methods).

± : Complete suppression.

+: Three-quarters suppression.

‡: Half suppression. ‡: Quarter suppression.

‡: No suppression.

graphy. As the result, R-2 is also composed of two sorts of compounds, namely L-rhamnose and hydroxyfatty acid identical with that of R-1. Accordingly, it is concluded that both glycolipids were composed of L-rhamnose and hydroxyfatty acid. Further study on the chemical structure, which will be presented elsewhere by SHIRAHATA *et al.*⁷⁾, proposed the defined structure for them, namely 2-O- α -L-rhamnopyranosyl- α -L-rhamnopyranosyl- β -hydroxydecanoyl- β -hydroxydecanoate for R-1 and L- α -rhamnopyranosyl- β -hydroxydecanoyl- β -hydroxydecanoate for R-2.

It is concluded from the above findings that R-1 was the same compound in which was isolated by F. G. JARVIS *et al.*¹⁾, while R-2 was newly isolated lipid, which was postulated as a precursor in the biosynthesis of the former by M. M. BURGER *et al.*³⁾

3. Culture Condition for Rhamnolipids Production

The production of the rhamnolipids was discovered first in the medium in which *n*-paraffin was used as the sole source of carbon described above. Therefore, other carbon sources were compared with it in subsequent experiments. As shown in Table 1, *n*-paraffin or glycerol was the preferable carbon source for the production of rhamnolipids. The total amount of rhamnolipids production was 8.5 mg/ml at the maximum. In the other experiment, the respective amount of R-1 and R-2 was determined after they were separated each other on thin-layer chromatography. The result showed that R-2 occupied about 40 % of total amount of rhamnolipids.

4. Biological Activities of Rhamnolipids

The minimal inhibitory concentration of R-1 and R-2 are listed in Table 2. As shown in Table 2, the biological activity of R-2 was more substantial against some gram-positive bacteria and *Proteus vulgaris* than that of R-1. It is of interest that the monorhamnosyl compound is more active than the dirhamnosyl compound.

Mycoplasmacidal and antiviral activities of these rhamnolipids are shown in Tables 3 and 4. Both R-1 and R-2 were active against *M. laidlawii* but little activity was observed against *M. gallisepticum*.

Antiviral activity of R-1 and R-2, was also observed at the concentration of about 10 mcg/ml. The acute toxicities of R-1 and R-2 (emulsions with liquid paraffin) to mice by intraperitoneal injection were as follows; LD₅₀ >400 mg/kg of R-1 and 117.5 mg/kg of R-2, respectively.

References

- 1) JARVIS, F. G. & M. J. JOHNSON: A glyco-lipide produced by *Pseudomonas aeruginosa*. J. Am. Chem. Soc. 71: 4124~4126, 1949
- 2) EDWARDS, J. R. & J. A. HAYASHI: Structure of a rhamnolipid from *Pseudomonas aeruginosa*. Arch. Biochem. Biophys. 111: 415~421, 1965
- 3) BURGER, M. M.; L. GLASER & R. M. RURTON: The enzymatic synthesis of a rhamnose-containing glycolipid by extracts of *Pseudomonas aeruginosa*. J. Biol. Chem. 238: 2595~2602, 1963
- 4) SUZUKI, T.; K. TANAKA, I. MATSUBARA & S. KINOSHITA: Trehalose lipid and α -branched- β -hydroxy fatty acid formed by bacteria grown on *n*-alkanes. Agr. Biol. Chem. 33: 1619~1627, 1969
- 5) HODGE, J. E. & B. T. HOFREITER: Methods in carbohydrate chemistry (R. L. WHISTLER, M. L. WOLFORM ed.) Vol. 1 p. 388~390 Academic Press, 1962
- 6) DUBOIS, M.; K. A. GILLES, J. K. HAMILTON, P. A. REBERS & F. SMITH: Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350~356, 1956
- 7) SHIRAHATA, K.; T. IDA & I. MATUBARA: The structure of a new rhamnolipid produced by *Pseudomonas aeruginosa*. in preparation